

Purification and properties of the phenprocoumon-induced decarboxyfactor x from bovine plasma

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PURIFICATION AND PROPERTIES OF THE PHENPROCOUMON-INDUCED DECARBOXYFACTOR X FROM BOVINE PLASMA

A COMPARISON TO NORMAL FACTOR X

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Summary

1. By a procedure involving adsorption to barium sulfate, chromatography on DEAE-Sephadex and QAE-Sephadex and preparative polyacrylamide gel electrophoresis, decarboxyfactor X was purified from plasma of phenprocoumon-treated cows. No contaminants could be detected in the final preparation by polyacrylamide gel electrophoresis and zone-electrophoresis.

2. The molecular weight of decarboxyfactor X, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis is approximately 55 000, which is equal to that of factor X. The protein consists of two polypeptide chains with molecular weights of 44 000 and 17 000.

3. Decarboxyfactor X has antigenic determinants in common with normal factor X.

4. The amino acid composition and aminoterminal amino acids of normal factor X and decarboxyfactor X are identical.

5. Less than one residue of γ -carboxyglutamate could be detected per mole of decarboxyfactor X.

6. In the absence of Ca^{2+} , normal factor X has a slightly higher electrophoretic mobility than decarboxyfactor X. In the presence of Ca^{2+} the mobility of factor X decreases considerably while the mobility of decarboxyfactor X remains unaltered.

Introduction

In several laboratories a decarboxyprothrombin present in plasma of dicoumarol-treated cattle has been extensively purified and characterized

[1-4]. The structural comparison of normal and dicoumarol-induced prothrombin has been the subject of intensive investigations in recent years [5-9]. It is now clear that prothrombin contains a number of γ -carboxyglutamate residues in the aminoterminal region, whereas glutamate residues are found in the aminoterminal region of decarboxyprothrombin.

These results and experiments on the biosynthesis of prothrombin [10-12] confirm the earlier suggestion of Hemker et al. [13] that decarboxyprothrombin is a precursor of normal prothrombin, which accumulates because of a decreased turnover of the vitamin K-dependent conversion of decarboxyprothrombin into prothrombin.

Since there are four vitamin-K dependent coagulation factors, one can postulate the existence of four decarboxy blood coagulation factors. We reported earlier the demonstration of both decarboxyfactor IX and decarboxyfactor X [14,15]. In this paper we describe the purification and partial characterization of decarboxyfactor X. The purification of decarboxyfactor X is of particular interest because it may be a suitable tool in studying the role of Ca^{2+} in the activation of factor X and their role in prothrombinase, especially on the proteolytic activity of factor X_a .

Materials

Normal bovine factors II, IX, and X used for the immunization of rabbits were purified according to the method of Reekers [16]. Common inorganic chemicals (reagent grade), barium sulfate, acrylamide, N',N' -methylene bisacrylamide and benzamidinium hydrochloride were obtained from Merck, Darmstadt, G.F.R. Several other types of BaSO_4 tested gave the same results with minor quantitative differences. Marcoumar was a kind gift of Dr. M. Kunz (Hoffmann-La Roche, Basle, Switzerland). DEAE-Sephadex and QAE-Sephadex (Pharmacia, Uppsala, Sweden) were equilibrated according to the instructions supplied by the manufacturers. Agarose was a product of Koch-Light Laboratories Ltd. Aluminum hydroxide moist gel was purchased from British Drug Houses Ltd. Sodium dodecyl sulfate (Koch-Light Laboratories Ltd.) was recrystallized from ethanol. Bovine serum albumin, glutamate dehydrogenase, ovalbumin, carbonic anhydrase, lysozyme, myoglobin, α -chymotrypsin-A, soybean trypsin inhibitor, diisopropylfluorophosphate and dansylchloride were obtained from Sigma Chemical Co.

Reference dansylamino acids were supplied by Calbiochem A.G. Polyamide layer sheets were obtained from Merck. Vitamin K and heparin were purchased from Hoffmann-La Roche.

Methods

Preparation of rabbit antbovine factor II and IX antiserum

Antisera were prepared in rabbits by injection of purified preparations of bovine factor II and factor IX [15]. Antisera were adsorbed with barium sulfate (100 mg/ml) for 30 min at room temperature and heat inactivation was performed for 30 min at 56°C in order to remove procoagulant activity. The adsorbed and inactivated antisera were centrifuged for 1 h at $105\,000 \times g$ at

4°C. Sodium merthiolate (0.01% final concentration) was added before storage at -20°C.

Preparation of rabbit antibovine factor X antiserum

For the immunization of rabbits, we used a factor X₂ preparation purified as described under Methods. The first intramuscular injection was given at four different places with 1.0 ml factor X₂ (0.2 mg/ml) mixed with 1.0 ml complete Freund's adjuvant. A second and third intramuscular injection of 1.0 ml factor X₂ (0.2 mg/ml) mixed with 1.0 ml incomplete Freund's adjuvant was given after intervals of 3 weeks. Plasmaphoresis was performed after the second month. Antisera were treated as described above.

Gel electrophoresis

Analytical polyacrylamide gel electrophoresis was performed according to Ornstein [17], using a Tris · HCl · glycinate buffer system as described by Davis [18]. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) was carried out according to Weber and Osborn [19]. Bovine serum albumin, glutamate dehydrogenase, ovalbumin, carbonic anhydrase, α -chymotrypsinogen A, myoglobin and lysozyme were used as markers for the molecular weight estimation, using molecular weights given by Weber and Osborn [19]. The gels were stained according to Fairbanks [20]. Equipment for analytical polyacrylamide gel electrophoresis was obtained from Buchler Instruments (Poly-analyst).

Agarose gel electrophoresis was carried out in 1.5 mm thick 1% agarose gels in 0.05 M barbital buffer pH 8.6 containing 2.0 mM calcium-lactate or 2.0 mM EDTA by the method of Johansson [21]. Electrophoresis was run at 15 V per cm on a glass plate and cooled by water of 10°C. The gels were fixed in 10% trichloroacetic acid and stained with Coomassie Brilliant Blue G (Sigma Chemical Co.) in 10% acetic acid. A LKB Multiphor 2117 apparatus was used.

Immunochemical methods

Decarboxyfactor X was immunochemically quantitated with the technique of Laurell [22]. Electrophoresis was performed (10 Volts/cm for 3 h) into a 1% agarose gel containing 1% anti-bovine factor X antiserum, using 0.05 M barbital buffer pH 8.6 in the presence of 2.0 mM calcium-lactate. As standard was used a plasma obtained from a cow under deep anticoagulation (factor X activity less than 5%). One unit is arbitrarily defined as the concentration of factor X-related antigen in 1.0 ml of standard plasma.

The crossed immunoelectrophoresis was carried out according to Laurell [23] on glass plates (10 × 20 cm). Agarose gel 1% in 0.05 M barbital buffer pH 8.6 containing 2.5 mM calcium-lactate or EDTA in the first dimension was used. After the first dimension run, the agarose gel 0.5 cm above the slit was replaced by 1% agarose containing 0.5% anti-bovine factor X antiserum and 1.0 mM calcium-lactate. Electrophoresis in the second dimension was continued for 4 h at 10 V/cm. After the completion of the electrophoresis the gel was washed overnight in saline-phosphate solution, then washed in distilled water for 2 h, dried and stained with 0.025% Coomassie Brilliant Blue R in methanol/water/acetic acid (450 : 450 : 100, v/v). Analyses were performed in

a LKB Multiphor 2117 apparatus. Double immunodiffusion was carried out according to Ouchterlony [24].

Coagulation factor assay

Coagulation times specifically dependent on the concentrations of factor II, VII, and X were obtained by mixing in a test tube at 37°C 0.1 ml bovine plasma, artificially deficient in either factor II, VII, or X, 0.1 ml sample and 0.1 ml bovine brain thromboplastin, prepared according to Owren [25]. The coagulation reaction was started by adding 0.1 ml CaCl_2 solution (33 mM). Coagulation factor concentrations are expressed as a percentage of the content in a normal bovine plasma pool, obtained from 20 cows. The concentrations were calculated from the coagulation times in specific tests as described by Hemker et al. [26]. It is defined that 1 ml bovine plasma with a blood coagulation factor activity of 100% contains 1 Unit of each of the factors.

Preparation of normal bovine factor X

Bovine factor X was prepared with the method of Jackson [27] with slight modifications. The two forms of factor X, designated factor X_1 and factor X_2 [28] were separated by chromatography on QAE-Sephadex. Therefore, factor X eluted from the DEAE-Sephadex column was dialyzed against 0.05 M trisodium citrate, pH 7.0, and applied to a column of QAE-Sephadex equilibrated with the same buffer. Factor X was eluted with a linear gradient of 0.10–0.35 M trisodium citrate, pH 7.0. The factor X_1 - and factor X_2 -containing fractions were pooled separately and concentrated in a Diaflow ultrafiltration cell equipped with a PM-10 membrane. The concentrate was adjusted to pH 8.1 with 1.0 M Tris · HCl, pH 8.6. Diisopropylfluorophosphate (1.0 M in 2-propanol) was added to a final concentration of 1 mM and incubated for 60 min at 4°C. After dialysis against 0.025 M Tris · HCl buffer, pH 7.4, containing 0.12 M NaCl during 48 h at 4°C with four changes, the factor X_1 and X_2 preparations were stored at –80°C.

Induction of decarboxyfactor X in the cow

Four cows, weighing about 500 kg each, were orally given 600 mg Marcoumar the first day, followed by 200 mg daily for 4–6 days. Blood samples were drawn from a jugular vein each day during the treatment and collected into polyethylene tubes (50 ml) containing 0.8 ml of 0.7 M trisodium citrate solution. The effect of Marcoumar was monitored by the assay of factor II, VII, and X activity and by crossed immunoelectrophoresis of plasma samples. At a factor X activity of about 10% of the normal activity, 5 l of blood were collected from a jugular vein of each cow in 1-l polyethylene bottles containing 100 ml 0.1 M sodium oxalate, 10 mg heparin, 10 g benzamidine hydrochloride and 5 mg crude soybean trypsin inhibitor. Afterwards, the animals were given 50 mg vitamin K each. Plasma was obtained by centrifugation in an MSE Mistral 61 centrifuge at 2500 rev./min during 20 min at 4°C.

Preparation of decarboxyfactor X

Bovine decarboxyfactor X was prepared as follows:

Step 1: Removal of normal factor X. Marcoumar plasma (21 l) was stirred with 30 g moist gel $\text{Al}(\text{OH})_3$ in 150 ml water during 10 min at room temperature. The $\text{Al}(\text{OH})_3$ was removed by centrifugation at $2000 \times g$ for 10 min at 4°C .

Step 2: Adsorption of decarboxyfactor X on barium sulfate. The supernatant (21 150 ml) was stirred with 400 g barium sulfate suspended in a minimal amount of 0.45% (w/v) NaCl in 1 mM Tris buffer pH 7.5 for 30 min at 4°C . BaSO_4 was removed by centrifugation at $1500 \times g$ for 5 min and washed three times with 1 l of the same buffer. Protein was eluted from the BaSO_4 with 1050 ml of 0.12 M trisodium citrate in 0.05 M Tris · HCl buffer, pH 5.8 containing 0.01 M benzamidine-HCl and 40 mg purified soybean trypsin inhibitor per litre, by stirring at 4°C for 30 min. This step was repeated once.

Step 3: DEAE-Sephadex adsorption and elution. The combined BaSO_4 eluates (2100 ml) were diluted with an identical volume of distilled water and adjusted to pH 7.1 with 1.0 M NaOH. A slurry settled by gravity of DEAE-Sephadex (80 ml) equilibrated in 0.06 M trisodium citrate in 0.05 M Tris · HCl buffer, pH 7.1, was added to the eluate and stirring was continued for about 30 min at room temperature until all factor X-related antigen was bound to the DEAE-Sephadex. In addition, the DEAE-Sephadex was washed with 1 l of 0.1 M NaCl in 0.02 M potassium phosphate buffer of pH 6.8. The DEAE-Sephadex was packed into a column (45×2.5 cm) onto a 3 cm high DEAE-Sephadex layer equilibrated in the final washing buffer. Decarboxyfactor X was eluted using a linear gradient of 0.1–0.6 M NaCl in a 0.02 M potassium phosphate buffer of pH 6.8 containing 1 mM benzamidine hydrochloride. Each chamber contained 250 ml of the solution. The effluent of the DEAE-Sephadex column was monitored by rocket immunoelectrophoresis according to Laurell [22] using a monospecific anti-bovine factor X antiserum.

Step 4: QAE-Sephadex chromatography. The decarboxyfactor X fractions from the DEAE-Sephadex column were pooled, dialyzed overnight against 0.05 M trisodium citrate, 1 mM benzamidine hydrochloride pH 7.0. The same QAE-Sephadex chromatography procedure as described for factor X purification was followed. Decarboxyfactor X-containing fractions were identified by rocket immunoelectrophoresis.

Step 5: Preparative polyacrylamide gel electrophoresis. The decarboxyfactor X-containing fractions from the QAE-Sephadex column were dialyzed against 2 mM Tris · HCl · glycinate buffer, pH 8.7, 10 mM in benzamidine hydrochloride, and concentrated in a Diaflow ultrafiltration cell equipped with a PM-10 membrane (Amicon Corp. model 202). Generally 40 mg of protein in 3–4 ml dialysis buffer containing 20% (v/v) sucrose were subjected to preparative polyacrylamide gel electrophoresis on a gel bed of 10 cm in the LKB 7900 Uniphor apparatus. Fractions of 3.0 ml were collected. The elution buffer was pumped at a flow rate of 30 ml/h. Electrophoretic conditions were 700 V and 18 mA. Composition of the gel solutions: solution A, 30 g acrylamide and 0.9 g bisacrylamide dissolved in 100 ml water; solution B, 0.08 M Tris · glycine buffer, pH 8.7, and 4 mM benzamidine HCl; solution C, 140 mg ammonium persulfate dissolved in 100 ml water. Final gel solution was obtained by mixing 1 vol. A, 1 vol. B and 2 vol. C. According to the nomenclature of Hjerten [29] $T = 7.5\%$ and $C = 3\%$. Electrode chambers contained 20 mM Tris · glycine and

1 mM benzamidine hydrochloride at pH 8.7. The elution buffer was the same as the electrode buffer. The decarboxyfactor X containing fractions were pooled and concentrated in a Diaflow ultrafiltration cell equipped with a PM-10 membrane (Amicon Corp. Model 202). The concentrate was adjusted to pH 8.1 with 1.0 M Tris · HCl pH 8.6. 1.0 M diisopropylfluorophosphate in 2-propanol was added to a final concentration of 10^{-3} M. After dialysis against 0.025 M Tris · HCl, pH 7.4 0.12 M NaCl during 48 h with four changes, the decarboxyfactor X preparation was stored at -80°C .

Determination of NH_2 terminal amino acids

Aminoterminal amino acids were determined qualitatively by the dansyl-chloride procedure of Gray [30]. Dansylamino acids were identified on polyamide sheets using the procedure of Weiner et al. [31].

Amino acid analysis

The amino acid composition was determined according to Spackman [32]. The proteins (0.5–1.5 mg) were hydrolyzed in 6 M HCl in evacuated Pyrex tubes at 110°C for 24 h. The analyses were performed on an LKB amino acid analyzer 3201 using the one-column system of Hamilton [33]. γ -Carboxyglutamate was determined according to a modification of the method of Zytkevich [34]. Prior to hydrolysis, proteins (1.0 mg) were completely reduced in $[^3\text{H}]$ diborane after 96 h of incubation at room temperature. The three identified $[^3\text{H}]$ diborane reduction products are: homoserine arising from the aspartic acid, 5-hydroxynorvaline arising from glutamic acid, and 5,5'-dihydroxyleucine arising from γ -carboxyglutamate. The specific activity of 5,5'-dihydroxyleucine is equal to half of the specific activity of homoserine and 5-hydroxynorvaline. The number of γ -carboxyglutamate residues was estimated from the calculated specific activity of dihydroxyleucine (Kop, J.M.M. and Hemker, H.C. (1977), unpublished.)

Results

Preparation of monospecific antisera against bovine factors II, IX, and X

Undiluted rabbit anti-bovine factor X, anti-bovine factor IX, and anti-bovine factor II antiserum were allowed to diffuse against undiluted bovine plasma (Fig. 1). Single precipitation lines were formed, showing a monospecificity of each of the antisera. A faint extra precipitation line is seen when normal bovine plasma reacts with the antifactor X antiserum. We have not been able to distinguish between the two possible explanations viz. (a) An antibody against a trace contaminant in the factor X preparation; (b) a derivative of factor X in the normal plasma preparation. This experiment shows that factor II, factor IX, and factor X have no antigenic determinants in common, because all reactions show non-identity of both antigens and antibodies.

Preparation of decarboxyfactor X

A representative preparation of decarboxyfactor X is summarized in Table I. Unlike decarboxyprothrombin and decarboxyfactor IX, decarboxyfactor X will adsorb quantitatively onto 10 mg $\text{Al}(\text{OH})_3/\text{ml}$ plasma. However, normal factor

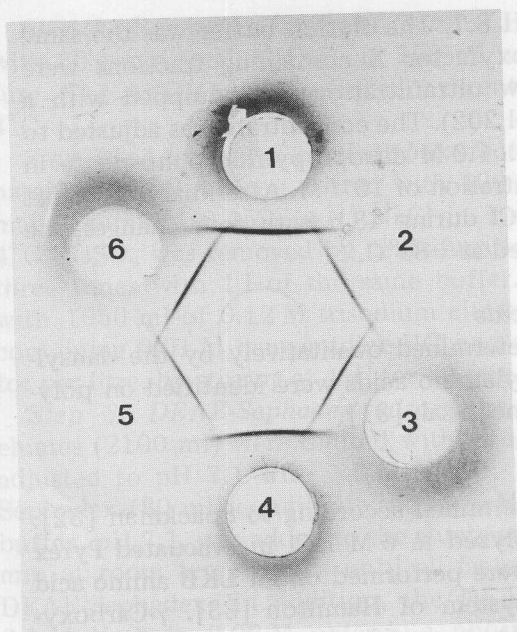


Fig. 1. Double immunodiffusion pattern of the antibody used. Centre well, normal bovine plasma; wells 1 and 4, anti-factor II antiserum; wells 2 and 5, anti-factor IX antiserum; wells 3 and 6, anti-factor X antiserum.

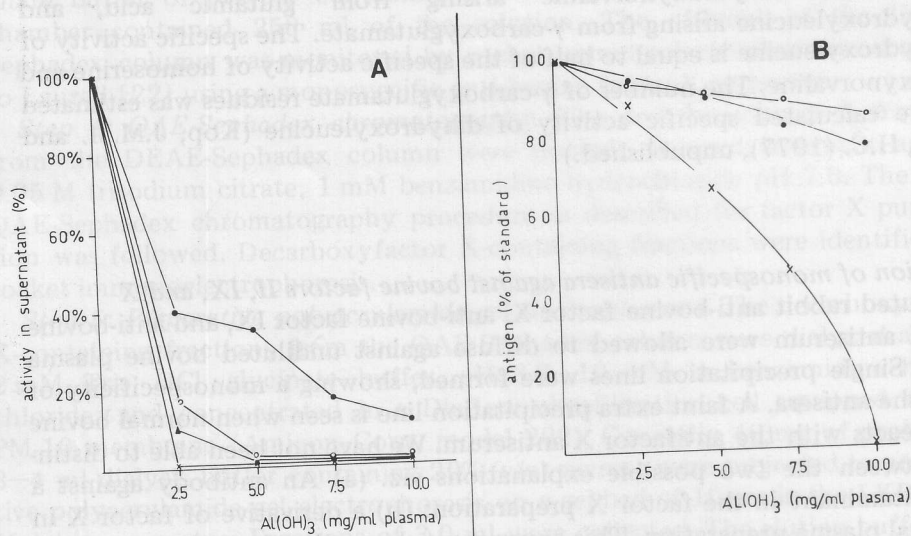


Fig. 2. Adsorption of factor II, IX, and X and their analog decarboxy factors onto $Al(OH)_3$ from oxalated plasma. A. Factor II (●), factor VII (▲), factor IX (○), and factor X (X) activity in the supernatant of plasma. Adsorption was performed at room temperature during 10 min. Clotting factor activity was measured by one stage assays as described under Methods. B. Decarboxyprothrombin (●), decarboxyfactor IX (○), decarboxyfactor X (X) concentration in the supernatant of normal bovine plasma (less than 5% of the normal factor II, factor IX, and factor X activities) after adsorption with different concentrations of $Al(OH)_3$. Adsorption was performed as described above. The concentrations of decarboxyfactor were determined by rocket immuno electrophoresis as described under Methods.

TABLE I
PURIFICATION OF DECARBOXYFACTOR X FROM COUMARIN OXALATED PLASMA
Results of a representative experiment starting with 21 l bovine plasma.

Fraction	Volume (ml)	Concentration (unit/ml)	Protein (mg/ml)	Spec. act. (unit/mg)	Yield (%)	Purification (-fold)
Plasma	21 000	1.0	77	0.013	100	1
BaSO ₄ eluate	2 100	2.5	5.9	0.4	25	33
DEAE-Sephadex	237	13.3	1.2	11.0	15	850
QAE-Sephadex	220	10.3	0.4	25.0	10	1900
Final preparation	112	4.3	0.1	43.0	2	3300

X will adsorb quantitatively onto 2.5 mg Al(OH)₃/ml plasma (Fig. 2). The supernatant of marcoumar plasma treated with 2.5 mg Al(OH)₃/ml plasma therefore contained most of the decarboxyfactor X, hardly any factor X and small amounts of the factors II and IX. It therefore is a suitable starting material for the preparation of decarboxyfactor X.

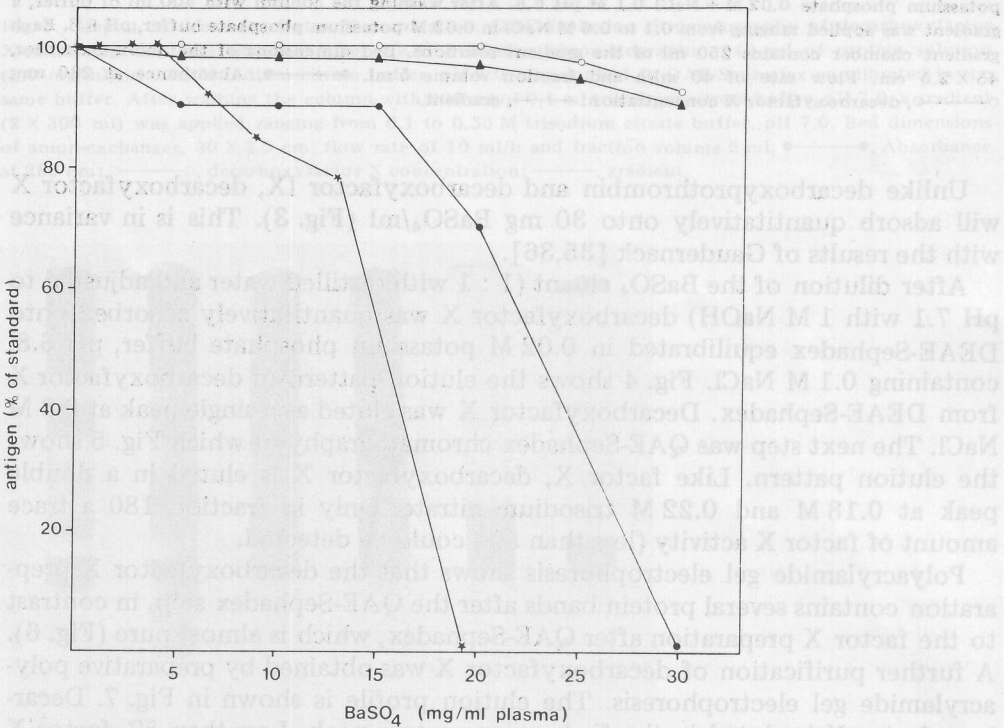


Fig. 3. Adsorption of decarboxyprothrombin, decarboxyfactor IX, decarboxyfactor X and factor X from oxalated bovine plasma onto BaSO₄. Decarboxyprothrombin (○), decarboxyfactor IX (▲), and decarboxyfactor X (●) concentration in the supernatant of bovine coumarin plasma (less than 5% of the clotting factor II, IX, and X activity) after adsorption during 30 min at room temperature with different concentrations of BaSO₄. Factor X concentration (★) in the supernatant of normal bovine plasma after adsorption with BaSO₄ under the same conditions as described above. The concentrations were determined by rocket immunoelectrophoresis as described under Methods.

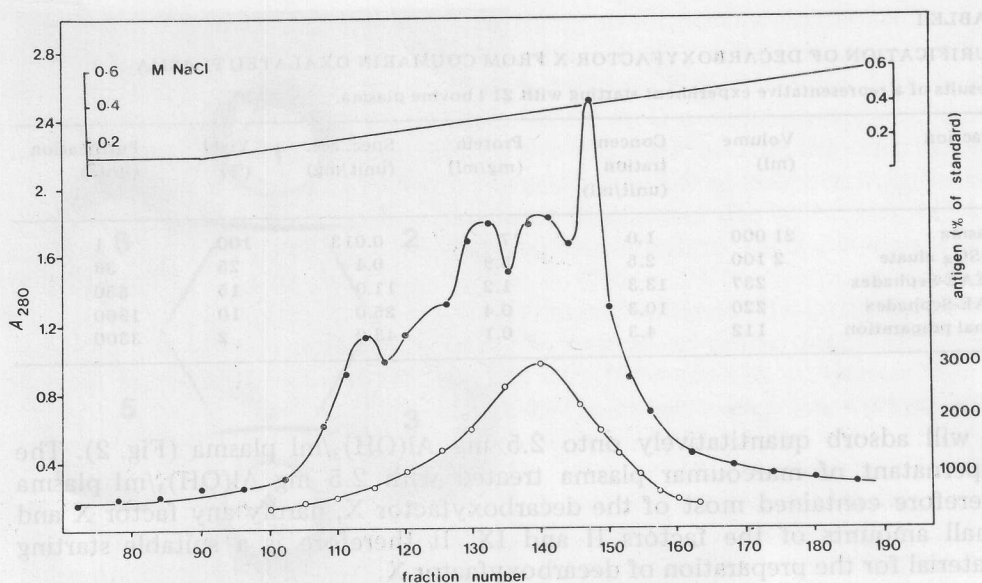


Fig. 4. Step 3 in decarboxyfactor X preparation. DEAE-Sephadex chromatography of BaSO_4 eluate in potassium phosphate 0.02 M + NaCl 0.1 M pH 6.8. After washing the column with 400 ml of buffer, a gradient was applied ranging from 0.1 to 0.6 M NaCl in 0.02 M potassium phosphate buffer, pH 6.8. Each gradient chamber contains 250 ml of the gradient solutions. Bed dimensions of the anion-exchanger, 45×2.5 cm. Flow rate of 40 ml/h and fraction volume 5 ml. ●—●, Absorbance at 280 nm; ○—○, decarboxyfactor X concentration; —, gradient.

Unlike decarboxyprothrombin and decarboxyfactor IX, decarboxyfactor X will adsorb quantitatively onto 30 mg BaSO_4/ml (Fig. 3). This is in variance with the results of Gaudernack [35,36].

After dilution of the BaSO_4 eluant (1 : 1 with distilled water and adjusted to pH 7.1 with 1 M NaOH) decarboxyfactor X was quantitatively adsorbed onto DEAE-Sephadex equilibrated in 0.02 M potassium phosphate buffer, pH 6.8, containing 0.1 M NaCl. Fig. 4 shows the elution pattern of decarboxyfactor X from DEAE-Sephadex. Decarboxyfactor X was eluted as a single peak at 0.3 M NaCl. The next step was QAE-Sephadex chromatography of which Fig. 5 shows the elution pattern. Like factor X, decarboxyfactor X is eluted in a double peak at 0.18 M and 0.22 M trisodium citrate. Only in fraction 180 a trace amount of factor X activity (less than 5%) could be detected.

Polyacrylamide gel electrophoresis shows that the decarboxyfactor X preparation contains several protein bands after the QAE-Sephadex step, in contrast to the factor X preparation after QAE-Sephadex, which is almost pure (Fig. 6). A further purification of decarboxyfactor X was obtained by preparative polyacrylamide gel electrophoresis. The elution profile is shown in Fig. 7. Decarboxyfactor X is eluted in the first major protein peak. Less than 5% factor X activity could be detected in the pooled decarboxyfactor X fractions. Analytical polyacrylamide gel electrophoresis shows no inhomogeneity in this final decarboxyfactor X preparation. It has the same electrophoretic mobility as factor X (gels 5 and 6 of Fig. 6, respectively). Like factor X, decarboxyfactor X is highly susceptible to proteolytic enzymes. A proper yield of decarboxyfactor X

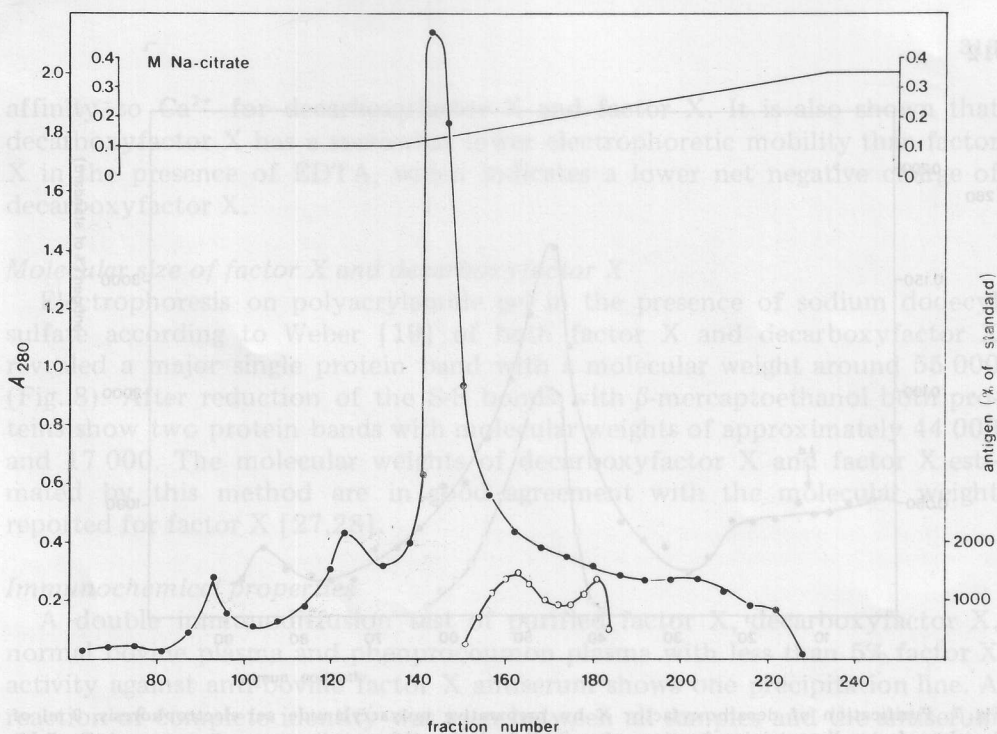


Fig. 5. Step 4 in decarboxyfactor X preparation. QAE-Sephadex chromatography of decarboxyfactor X-containing fractions obtained after DEAE-Sephadex chromatography. 240 ml of protein solution (1.0 mg/ml) in 0.05 M sodium citrate buffer, pH 7.0, was applied to QAE-Sephadex equilibrated in the same buffer. After washing the column with 600 ml of 0.1 M trisodium citrate buffer, pH 7.0, a gradient (2 × 300 ml) was applied ranging from 0.1 to 0.35 M trisodium citrate buffer, pH 7.0. Bed dimensions of anion-exchanger, 30 × 2.5 cm; flow rate of 10 ml/h and fraction volume 5 ml. ●—●, Absorbance at 280 nm; ○—○, decarboxyfactor X concentration; —, gradient.

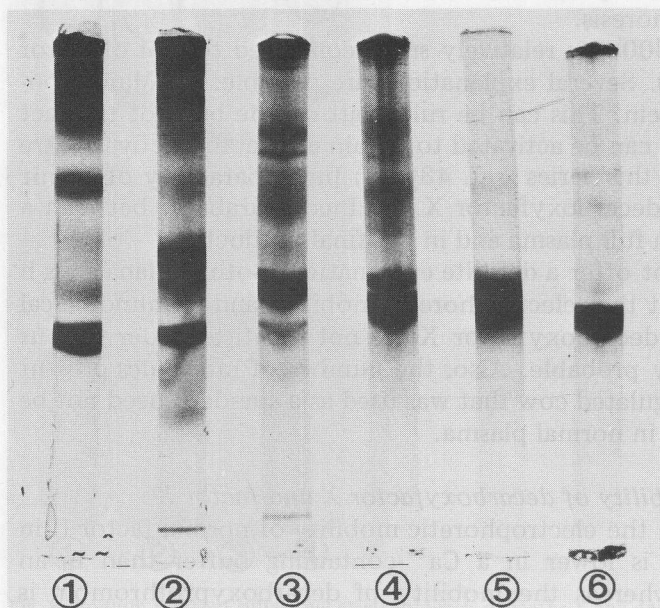


Fig. 6. Analytical polyacrylamide gel electrophoresis of normal bovine plasma and several fractions, obtained during the purification procedures. 1, Normal bovine plasma, 70 μ g; 2, BaSO_4 -eluate from decarboxyfactor X purification, 100 μ g; 3, decarboxyfactor X after DEAE-Sephadex, 45 μ g; 4, decarboxyfactor X after QAE-Sephadex, 50 μ g; 5, decarboxyfactor X after preparative polyacrylamide gel electrophoresis, 50 μ g; 6, factor X after QAE-Sephadex, 30 μ g.

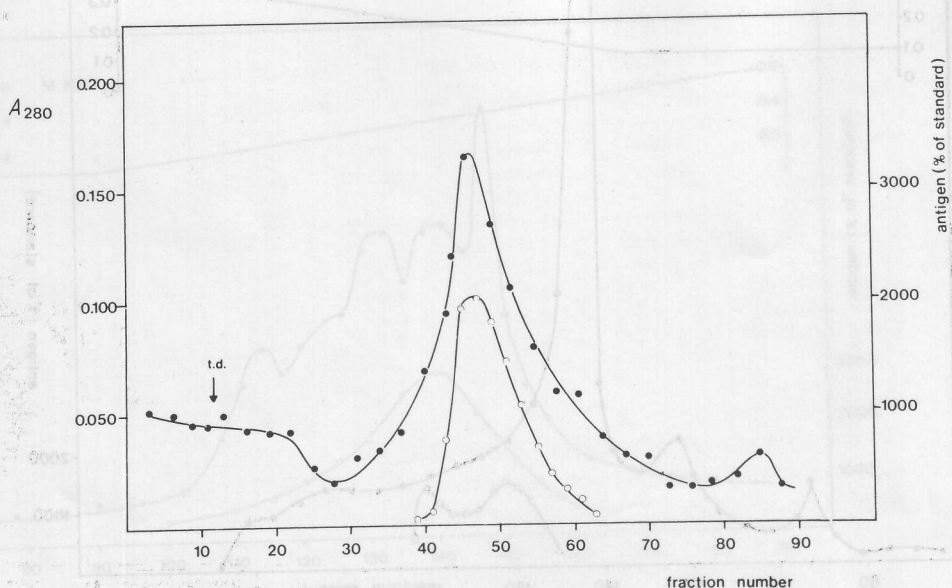


Fig. 7. Purification of decarboxyfactor X by preparative polyacrylamide gel electrophoresis. 3 ml of decarboxyfactor X-containing fractions after QAE-Sephadex (10 mg/ml) were applied on a gel ($T = 7.5\%$ and $C = 3\%$) [29] of 10 cm length. Fraction volume 3.0 ml. ●—●, Absorbance at 280 nm; ○—○, decarboxyfactor X concentration; t.d., tracking dye. Detailed experimental conditions are described under Methods.

could only be obtained by adding benzamidine hydrochloride to all buffers. Omission of benzamidine in the last purification step results in the appearance of three different degradation products of decarboxyfactor X, as judged by polyacrylamide gel electrophoresis.

The final purification (3300×) is relatively small compared to that of factor X, which can reach 10 000. Several explanations are possible: (a) Major contamination by another protein. This can be ruled out on the basis of the fact that 90% of the preparation can be activated to obtain a titratable active centre as is shown in article 3 of this series (ref. 43). (b) Incomparability of a unit of factor X and a unit of decarboxyfactor X. (c) Incomparability between a unit of decarboxyfactor X in full plasma and in the final product.

At the moment we cannot offer a definite explanation. Both explanations b and c are based on the fact that electrophoretic mobility and immunological reactivity of factor X and decarboxyfactor X are not identical under various circumstances, which is very probable. Also, the number of molecules present in the plasma of an anticoagulated cow that was used as a standard need not be the same as that of factor X in normal plasma.

Relative electrophoretic mobility of decarboxyfactor X and factor X

As shown by Stenflo [4] the electrophoretic mobility of normal factor II in agarose gel electrophoresis is lower in a Ca^{2+} -containing buffer than in an EDTA-containing buffer, whereas the mobility of decarboxyprothrombin is unaffected by Ca^{2+} . Agarose gel electrophoresis of factor X and decarboxyfactor X in the presence of Ca^{2+} or EDTA demonstrates identical differences in

affinity to Ca^{2+} for decarboxyfactor X and factor X. It is also shown that decarboxyfactor X has a somewhat lower electrophoretic mobility than factor X in the presence of EDTA, which indicates a lower net negative charge of decarboxyfactor X.

Molecular size of factor X and decarboxyfactor X

Electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate according to Weber [19] of both factor X and decarboxyfactor X revealed a major single protein band with a molecular weight around 55 000 (Fig. 8). After reduction of the S-S bonds with β -mercaptoethanol both proteins show two protein bands with molecular weights of approximately 44 000 and 17 000. The molecular weights of decarboxyfactor X and factor X estimated by this method are in good agreement with the molecular weight reported for factor X [27,28].

Immunochemical properties

A double immunodiffusion test of purified factor X, decarboxyfactor X, normal bovine plasma and phenprocoumon plasma with less than 5% factor X activity against anti-bovine factor X antiserum shows one precipitation line. A reaction of complete identity was seen between all samples and the antiserum (Fig. 9).

When purified factor X and purified decarboxyfactor X were analyzed by crossed immunoelectrophoresis in calcium-containing buffer, single symmetri-

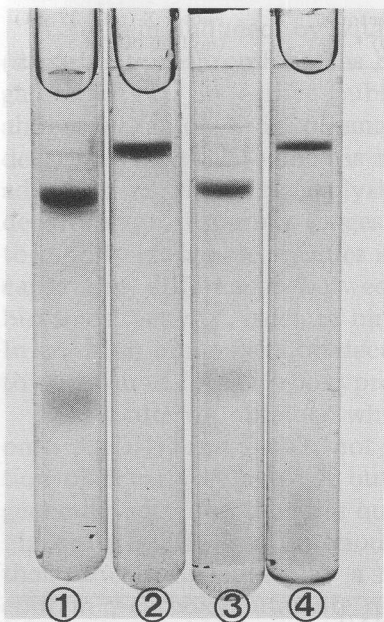


Fig. 8. Sodium dodecyl sulfate gel electrophoresis of factor X and decarboxyfactor X. Gel electrophoresis system of Weber and Osborn [19]. 1, Factor X, disulfide reduced; 2, factor X; 3, decarboxyfactor X, disulfide reduced; 4, decarboxyfactor X. Approximate loads for each gel, 15–20 μg of protein.

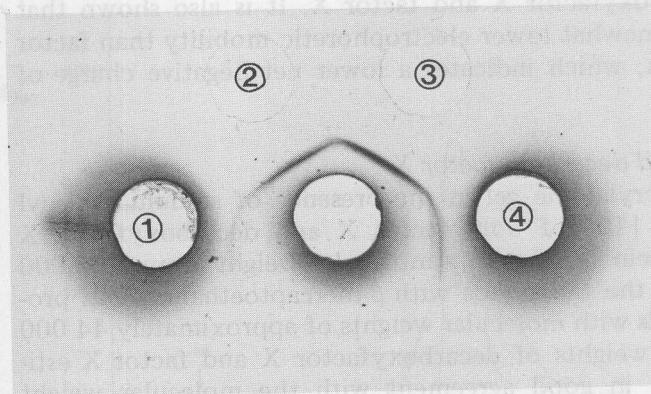


Fig. 9. Double immunodiffusion. Centre well contained anti-bovine factor X antiserum. Well 1, coumarin bovine plasma; 2, decarboxyfactor X; 3, factor X; 4, normal bovine plasma.

cal arcs were obtained with both proteins showing a higher electrophoretic mobility for decarboxyfactor X. With this technique factor X could not be detected in the decarboxyfactor X preparation. In the presence of EDTA the precipitation arc of decarboxyfactor X is at the same position as that found for factor X. The immunoelectrophoretic patterns obtained with the purified proteins were not different from those obtained with unfractionated plasma.

TABLE II

AMINO ACID COMPOSITION OF FACTOR X AND DECARBOXYFACTOR X

Amino acid	Factor X (mol/50 000) *	Decarboxyfactor X (mol/50 000) *	Factor X (refs. 37, 41) (mol/50 000) *
Lysine	20	21	21
Histidine	11	11	10
Arginine	25	25	25
Aspartic acid	40	42	40
Threonine	28 **	28	30
Serine	31 **	30	29
Glutamic acid	61	61	61
Proline	20	18	20
Glycine	42	37	41
Alanine	31	29	31
Half-cystine	19	20	24
Valine	20	22	24
Methionine	6	6	6
Isoleucine	11	13	11
Leucine	33	29	32
Tyrosine	11	11	9
Phenylalanine	22	22	21
γ -Carboxyglutamic acid *** 1	12	<1	8 †
γ -Carboxyglutamic acid *** 2	13	<1	13 †

* Molecular weights do not include the carbohydrate content [37].

** Values determined by extrapolation to zero-time hydrolysis.

*** Values obtained after reduction with $[^3\text{H}]$ diborane and acid hydrolysis as described under Methods. 1 and 2 refer to factor X_1 and factor X_2 , respectively.

† Values taken from Neal et al. [41].

Amino acid analysis

The amino acid composition of decarboxyfactor X and factor X_{1,2} appeared to be identical within the experimental error (Table II), and is in good agreement with published data [28,37]. The composition given is the average of two different preparations of each of the proteins. The number of γ -carboxyglutamate residues was determined in decarboxyfactor X, factor X₁ and factor X₂ by a modification of the method of Zytkevich [34] as described in Methods. Less than one γ -carboxyglutamate residue was detectable per mol of decarboxyfactor X. No differences were found in the γ -carboxyglutamate content of factor X₁ and factor X₂ (Table II). These results are in close agreement with the number of γ -carboxyglutamate residues suggested from analysis of the N-terminal portion of the light chain, prepared from a mixture of factor X₁ and factor X₂ [38].

Aminoterminal amino acid analysis

Qualitative aminoterminal amino acid determination of decarboxyfactor X and factor X with a method according to Gray [30] showed a single intensive spot and several very weak spots by chromatography on polyamide sheets as described by Weiner et al. [31]. In both factor X and decarboxyfactor X the main spot was identified as dansylalanine. Fujikawa [28] reported the sequence of the NH₂-terminal region of the heavy chain to contain Trp-Ala-Ile-His. With the method of Gray [30] tryptophan degrades and, therefore, cannot be detected.

Discussion

A protein induced by vitamin K antagonists, analogous to prothrombin, (decarboxyprothrombin) has been demonstrated and isolated by many investigators [1-4]. In earlier publications we demonstrated the existence of an abnormal factor X in plasma of phenprocoumon-treated cattle (defined as decarboxyfactor X) which is ineffective in blood coagulation [14,15]. Recent advances in chemical analysis of the difference between prothrombin and decarboxyprothrombin suggest that the vitamin-dependent mechanism serves to modify glutamate residues into γ -carboxyglutamate residues [6,8]. This may cause the differences between prothrombin and decarboxyprothrombin in biological activity, calcium binding and adsorption to insoluble inorganic salts. In addition our results on decarboxyfactor X may represent a situation parallel to that found for decarboxyprothrombin.

The results we obtained when studying the adsorption of decarboxyfactor X onto Al(OH)₃ and BaSO₄ not only provided a useful first step for the purification of decarboxyfactor X but also raise a question of theoretical interest. It is generally accepted that the quantitative and selective adsorption of prothrombin onto BaSO₄ is a function of the γ -carboxyglutamate residues. It appears that other influences play a role as well, as decarboxyfactor X that does not contain γ -carboxyglutamate residues adsorbs at a slightly higher BaSO₄ concentration than does normal factor X. This result pertains to the interpretation of earlier results of Hemker et al. [13]. They described a protein that competitively inhibits tests of the prothrombin-time (c.q. Quick-time) type. From this

they concluded that in dicoumarol treatment abnormal prothrombin-like molecules, probably precursors of the normal factors, circulate. Later they localized the site of inhibition of the blood coagulation sequence at conversion of factor X and inferred that the inhibition is a factor X analog, i.e., decarboxy-factor X [40].

Therefore, in the light of the findings presented in this paper this claim can not be challenged on the bases that the inhibitor was reported to be adsorbed onto $\text{Al}(\text{OH})_3$ and BaSO_4 .

Comparison of the electrophoretic mobility of prothrombin and factor X may reveal a larger net negative charge on factor X. The more anodical position of decarboxyfactor X relative to that of decarboxyprothrombin also indicates the same difference in net negative charge [14]. These facts may explain the higher affinity of decarboxyfactor X towards insoluble barium salts as compared with decarboxyprothrombin. The isolation procedure of decarboxy-factor X described in this paper yields a preparation which is fairly pure as judged by several criteria, e.g. gel electrophoresis and aminoterminal amino acid analysis. The heterogeneity of decarboxyfactor X, as demonstrated in the QAE-Sephadex elution profile, is of interest. It is unlikely that this phenomenon is due to the presence of factor X, because the factor X activity is far less than would be expected from the amount of precipitable antigen.

It was reported by Neal et al. [41] that factors X_1 and X_2 differ in their γ -carboxyglutamate content (8 and 13 residues, respectively) and that these differences may be accounted for by the different behaviour on anion-exchange chromatography. However, in our hands an equal amount of γ -carboxyglutamate residues was found in both factor X_1 and factor X_2 . Moreover, decarboxyfactor X, which contains less than one residue of γ -carboxyglutamate, also shows heterogeneity on anion-exchange chromatography under certain conditions.

Decarboxyfactor X is very similar to factor X on the basis of molecular weight, polypeptide chain composition, amino acid composition, aminoterminal amino acid, immunochemical reaction and its behaviour on polyacrylamide gel electrophoresis. Furthermore, it is shown that the purified decarboxyfactor X and the native protein in unfractionated plasma are similar as they have identical immunochemical and electrophoretic properties.

Based on amino acid analysis the difference between factor X and decarboxyfactor X in biological activity and Ca^{2+} binding may be explained by the difference found in γ -carboxyglutamate content. Ca^{2+} binding properties of decarboxyfactor X and their role in the conversion of the zymogen in a serine protease, analogous to factor X_a , are reported in the following papers [42,43].

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